Prior induction of heme oxygenase-1 with glutathione depletor ameliorates the renal ischemia and reperfusion injury in the rat

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Abstract Heme oxygenase (HO)-1 catalyzes the rate-limiting step in heme degradation releasing iron, carbon monoxide, and biliverdin. Induction of HO-1 occurs as an adaptive and protective response to oxidative stress. Ischemia and reperfusion (IR) injury seems to be mainly caused by the oxidative stress. In this study, we have examined whether prior induction of HO-1 with buthionine sulfoximine (BSO), a glutathione (GSH) depletor, affects the subsequent renal IR injury. BSO (2 mmol/kg body weight) was administered intraperitoneally into rats, the levels of HO-1 protein increased within 4 h after the injection. When BSO was administered into rats at 5 h prior to the renal 45 min of ischemia, the renal IR injury was assessed by determining the levels of blood urea nitrogen and serum creatinine, markers for renal injury, after 24 h of reperfusion. The renal injury was significantly improved as compared to the rats treated with IR alone. Administration of zinc-protoporphyrin IX, an inhibitor of HO activity, reduced the efficacy of BSO pretreatment on the renal IR injury. Our findings suggest that the prior induction of HO-1 ameliorates the subsequent renal IR injury.

Key words: Heme oxygenase-1; Oxidative stress; Ischemia–reperfusion; Renal injury; Glutathione; Buthionine sulfoximine

1. Introduction

Heme oxygenase (HO) is the rate-limiting enzyme that catalyzes the conversion of heme into biliverdin, carbon monoxide, and free iron [1]. Biliverdin is subsequently reduced by biliverdin reductase to form bilirubin, which scavenges reactive oxygen species. Two isoforms of HO have been extensively characterized: an inducible enzyme (HO-1) and a constitutive isoform (HO-2) [2]. Recently, a third isoform (HO-3) has been identified but its function is still unknown [3]. HO-1 is upregulated following various stimuli, including heme, heavy metals, cytokines, hormones, endotoxins, and heat shock, and is considered to be one of the most sensitive indicators of cellular oxidative stress [4,5].

It has been shown that HO-1 is induced in the kidneys in certain models of acute renal injury: glycerol-induced acute renal failure [6–8], nephrotoxic serum nephritis [6], and cisplatin nephrotoxicity [9]. Induced HO-1 protein was mainly localized in renal tubules, which were damaged regions, of rat models of rhabdomyolysis and nephrotoxic serum nephritis [6,7]. In a rat model of rhabdomyolysis, Nath's group reported that prior induction of HO-1 by preinfusion of hemoglobin prevented the renal failure and reduced mortality [6]. Under these conditions, administration of a specific inhibitor of HO activity worsened the renal damage further [6]. Hence, the stimulation of HO-1 activity with some non-toxic drug may represent a novel therapeutic approach in the amelioration of acute renal injury and other tissue insults.

It has been reported that HO-1 is induced by agents that are known to interact with or modify cellular glutathione (GSH) levels [10]. GSH is considered to be an important cellular antioxidant [11,12]. Therefore, it is tempting to speculate that the induced HO-1 expression by changing the intracellular redox state may protect the oxidative injury.

In this study, we investigated whether prior induction of HO-1 by buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase that is the rate-limiting enzyme in GSH biosynthesis, ameliorates the renal ischemia and reperfusion (IR) injury.

2. Materials and methods

2.1. Induction of acute renal injury

All animal experiments were performed following the institution's criteria for the care and use of laboratory animals. Male Wistar rats (200–250 g) were used. Animals were fed standard rodent chow and water ad libitum. Surgical operation was performed under intraperitoneal sodium pentobarbital (50 mg/kg body weight) anesthesia. For the renal ischemia, the right kidney was removed 3 weeks before the ischemia. Left renal ischemia was conducted for 45 min of occlusion of the renal artery and vein followed by 24 h of reperfusion. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush. GSH depletion was induced by intraperitoneal injection of BSO (2 mmol/kg body weight). Zinc-protoporphyrin IX (ZnPP), an inhibitor of HO activity, was twice injected into rats subcutaneously at 3 and 16 h before the start of ischemia.

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Abbreviations: BSO, L-buthionine-(*S,R*)-sulfoximine; HO, heme oxygenase; ZnPP, zinc-protoporphyrin IX; GSH, glutathione; BUN, blood urea nitrogen; SCr, serum creatinine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); IR, ischemia and reperfusion

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2.2. Assessment of renal injury

At the end of the experimental protocol, the rats were anesthetized with ether, the chest and peritoneal cavities were opened carefully, and blood was drawn from the heart. For the assessment of renal injury, blood urea nitrogen (BUN) and serum creatinine (SCr) levels were measured.

2.3. Biochemical analysis

Total GSH (reduced and oxidized forms) contents were measured with the GSH reductase–5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) recirculating assay [13]. The tissue (0.2 g) was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.5) at 0°C and mixed with 2.5 ml of 10% trichloroacetic acid solution. The mixture was centrifuged at $1500\times g$ for 15 min. One ml of supernatant was extracted three times with 2 ml of cold ether. Each assay contained 0.2 mM reduced NADPH, 0.6 mM DTNB, 0.6 U of GSH reductase and 100 μl of sample in a final volume of 1 ml. The reaction was started by the addition of GSH reductase. The rate of formation of reduced DTNB was followed at 415 nm.

2.4. Western blot analysis

Tissue extracts from kidneys were prepared as follows. Kidneys were homogenized in 10 volumes of homogenization buffer, which contained 20 mM Tris–HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and centrifuged at $8000\times g$ for 10 min at 4°C, and the resulting supernatants were used as tissue extracts. The extracts (100 μg protein/sample) were subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [14]. After electrophoresis, the fractionated proteins were electrophoretically blotted onto nitrocellulose filters. The membranes were first treated with 3% non-fat dry milk in TBST solution (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, and 0.05% Tween 20) for 1 h and then incubated with antibody against rat HO-1 or HO-2 (diluted 1000-fold, StressGen, Victoria, BC, Canada) in 3% non-fat dry milk in TBST solution for 1 h at room temperature. The filters were washed three times with TBST solution for 10 min each to remove any unbound antibodies and then were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) in 3% non-fat dry milk in TBST solution. After washing three times with TBST solution for 10 min each and once with 100 mM Tris–HCl (pH 9.5) solution containing 100 mM NaCl and 10 mM $MgCl_2$, the immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride as recommended by the supplier.

2.5. Histology

The rats were killed and the isolated kidneys were subjected to histological analysis. The kidneys were immediately fixed with 10% formalin, and were processed for paraffin embedding. 4-μm-thick paraffin sections were stained with hematoxylin and eosin.

2.6. Statistics

The data were expressed as mean ± S.D. for each group. Statistical analysis was performed by analysis of variance with a multiple comparison test (Bonferroni's multiple *t*-test). Differences were considered to be statistically significant when the P values were less than 0.05.

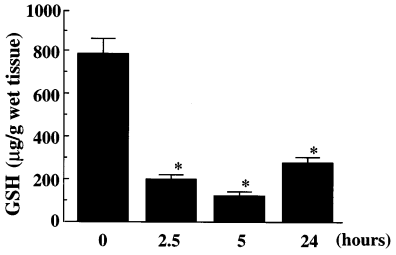

Fig. 1. Renal total GSH levels after BSO injection. BSO (2 mmol/kg body weight) was intraperitoneally injected into rats. At the indicated times, renal total GSH levels were determined as described in Section 2. Data represent the mean ± S.D. of five or six rats per group. $*P<0.05$, normal rats vs. BSO-treated rats.

3. Results

3.1. Effect of BSO on the renal total GSH levels

To determine the effect of BSO on the levels of total GSH (reduced and oxidized forms) in rat kidneys, we examined the time course of the changes in the total GSH content after the BSO injection (Fig. 1). Intraperitoneal administration of BSO (2 mmol/kg body weight) rapidly decreased the total GSH levels at 2.5 h after the injection (197.2 ± 25.4 μg/g wet tissue) and the levels were further reduced at 5 h (117.7 ± 23.6 μg/g wet tissue) as compared to the control levels (779.8 ± 76.8 μg/g wet tissue). The levels of total GSH seemed to be increased at 24 h (273.9 ± 27.9 μg/g wet tissue). These results showed that administration of BSO abruptly diminished the renal total GSH levels.

3.2. Induction of HO-1 protein in rat kidneys after IR

To determine the effect of renal IR on the expression of HO-1, we examined the time course of the levels of HO-1 protein in rat kidneys after the start of reperfusion following 45 min of ischemia (Fig. 2). Although HO-1 protein was not detected in rat kidneys treated with 45 min of ischemia alone, the band of HO-1 was first detected at 4 h and gradually increased to 24 h after the start of reperfusion. HO-1 protein was also undetectable in normal rat kidneys (data not shown). On the other hand, the levels of HO-2 protein were substantially unchanged during experimental periods.

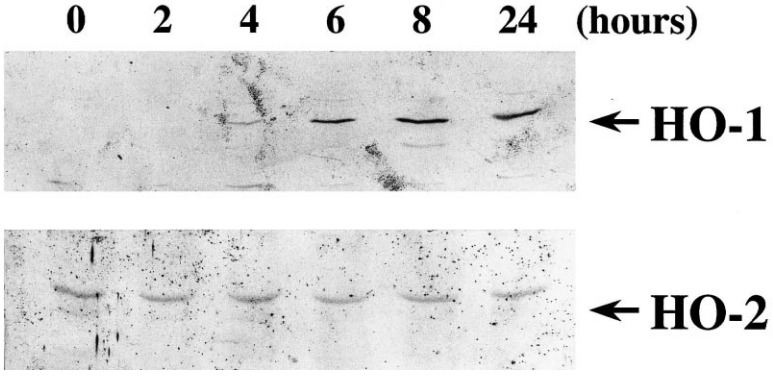

Fig. 2. Immunoblot analysis of HO-1 and HO-2 proteins in rat kidneys with IR. Rats underwent 45 min of ischemia followed by various times of reperfusion. At the indicated times after the start of reperfusion, the kidneys were isolated and the tissue extracts (100 μg/lane) were analyzed by Western blot analysis using antibodies against rat HO-1 and HO-2, respectively. 0 denotes the 45 min of ischemia alone.

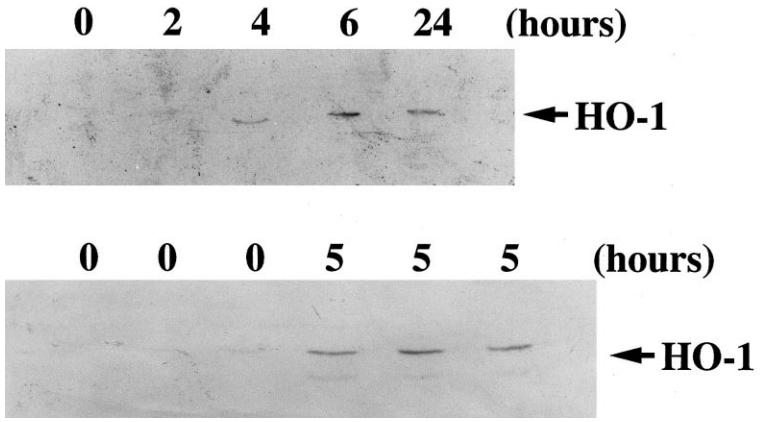

Fig. 3. Immunoblot analysis of HO-1 protein in rat kidneys after BSO administration. BSO (2 mmol/kg body weight) was injected intraperitoneally into rats. At the indicated times, the kidneys were isolated and the tissue extracts (100 μg/lane) were analyzed by Western blot analysis using antibody against rat HO-1. 0 denotes the untreated control rats. Lower gel showed that the kidney samples were isolated from three different rats in each experimental group.

3.3. Effect of BSO on the levels of HO-1 protein in rat kidneys

To determine the changes in the levels of HO-1 protein after BSO administration, we examined the time course of the levels of HO-1 protein in rat kidneys after BSO injection (Fig. 3). Intraperitoneal injection of BSO (2 mmol/kg body weight) obviously induced the HO-1 protein at 4 h after the injection and the high levels of HO-1 protein were sustained to 6–24 h. In addition, we confirmed that the expression of HO-1 protein was induced reproducibly at 5 h after the BSO administration. On the other hand, the levels of HO-2 protein were substantially unaffected by BSO administration (data not shown).

3.4. Effect of BSO pretreatment on the acute renal injury

BSO (2 mmol/kg body weight) was intraperitoneally administered into rats at 5 h prior to the renal ischemia, the levels of BUN and SCr, as markers for renal injury, were assessed at 24 h after the start of reperfusion following 45 min of ischemia (Fig. 4). The levels of BUN and SCr in rats treated with IR (BUN, 135 ± 39.9 mg/dl; SCr, 2.46 ± 0.91 mg/dl) were elevated as compared to the control rats (BUN, 21.5 ± 1.5 mg/dl; SCr, 0.63 ± 0.05 mg/dl). When BSO was administered into rats at 5 h prior to the renal ischemia, the renal injury (BUN, 47.0 ± 19.6 mg/dl; SCr, 0.78 ± 0.30 mg/dl) was obviously improved as compared to the rats treated with IR. On the other hand, administration of ZnPP, an inhibitor of HO activity, abolished the efficacy of BSO pretreatment (BUN, 132.6 ± 28.3 mg/dl; SCr, 2.57 ± 0.80 mg/dl). ZnPP treatment alone (BUN, 17.6 ± 2.2 mg/dl; SCr, 0.51 ± 0.05 mg/dl) did not show any differences as compared to the control rats. These results showed that BSO pretreatment ameliorated the renal IR injury. This improvement may be mediated directly or indirectly by HO-1 induction.

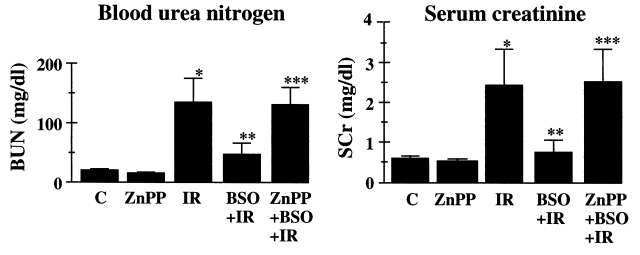

Fig. 4. Effect of BSO pretreatment on the subsequent renal IR injury in the rats. BSO (2 mmol/kg body weight) was intraperitoneally injected into rats at 5 h before the start of ischemia. After 45 min of ischemia followed by 24 h of reperfusion, BUN and SCr levels were determined. C, untreated control rats. Experimental details were described in Section 2. Data represent the mean ± S.D. of five or six rats per group. $*P < 0.05$, control rats vs. rats treated with IR; $**P < 0.05$, rats treated with IR vs. BSO-pretreated rats with IR; $***P < 0.05$, BSO-pretreated rats with IR vs. ZnPP- and BSO-treated rats with IR.

3.5. Histopathological analysis

To confirm the above findings, histopathological analysis was performed (Fig. 5). Histological data showed that there were no significant differences between control, BSO-, and ZnPP-treated rat kidneys. On the other hand, rat kidneys treated with IR clearly showed tubular damage, that is, dilation of the renal tubules with proteinaceous casts. These changes caused by IR were markedly improved with pretreatment of BSO. However, administration of ZnPP prior to the BSO treatment eliminated the therapeutic effects of BSO for IR injury.

4. Discussion

Cells primed by mild stress transiently induce stress proteins and thereby develop tolerance to the next severe stress. Induction of HO-1 in response to various oxidative insults has been shown to be implicated in a cytoprotective mechanism to prevent cells and tissues from further oxidative injury. In view of its important functions, there has been great interest in assessing the potential use of HO-1 as a therapeutic target for various oxidative injuries [6,15,16]. Therefore, if we can use non-toxic drugs to induce HO-1 in advance, it seems to be a clinically useful tool. Furthermore, to clarify the molecular mechanism of such drugs leads to the development of more useful medicine. In this study, our findings showed that the prior induction of HO-1 with BSO ameliorated the renal ischemic injury. BSO concentration used in this experiment did not affect any toxic influence on the normal renal function. Under this condition, the expression of HO-1 might be induced by changing the redox state without harmful effects.

Although several findings have been reported, the mecha-

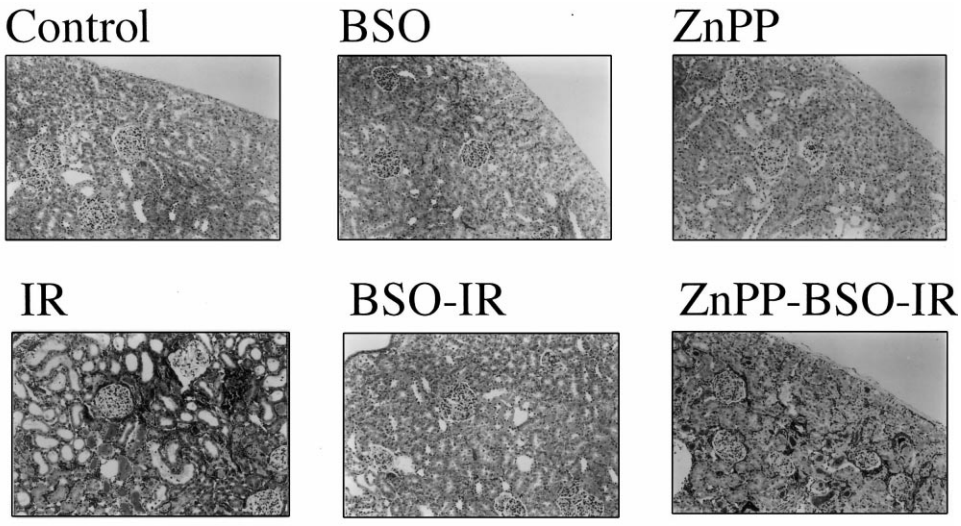

Fig. 5. Histopathological analysis of rat kidneys. Control, untreated rat kidneys. Experimental details were described in Section 2. These figures show the cortex including glomeruli. Hematoxylin and eosin, magnification ×40.

nisms behind HO-1-mediated cytoprotection are not fully understood. The end-products of heme degradation, including biliverdin, bilirubin and CO, show important physiological roles. Both biliverdin and bilirubin are efficient peroxyl radical scavengers that possess potent antioxidant properties [17,18]. Because the postischemic damage is at least in part considered to be related to the generation of free oxygen radicals, both bilirubin and biliverdin may play a protective role against reactive oxygen species generated by renal IR. In addition, CO is a potent activator of soluble guanylate cyclase [19] and a vasodilator substance [20]. It has been recently reported that inhaled CO mitigates lung IR injury in mice [21] and CO also protects against hepatobiliary dysfunction in endotoxin-treated rat liver [22]. Therefore, the beneficial effects of HO-1 preinduction with BSO may be derived from production of bilirubin and CO. In addition to the HO-1 induction, GSH depletor may induce a number of acute phase proteins and antioxidant enzymes.

Tolerance induction in the kidney by BSO is a promising novel strategy for preventing the critical problems associated with the IR injury. Further studies that clarify the mechanism of tolerance induction will provide clinically useful strategies against tissue injury induced by the oxidative stress.

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